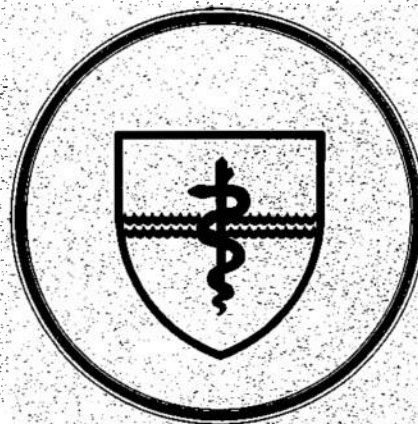


**NAVAL SUBMARINE MEDICAL
RESEARCH LABORATORY
SUBMARINE BASE, GROTON, CONN.**



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BONE CO_2 -TITRATION CURVES
IN ACUTE HYPERCAPNIA

by

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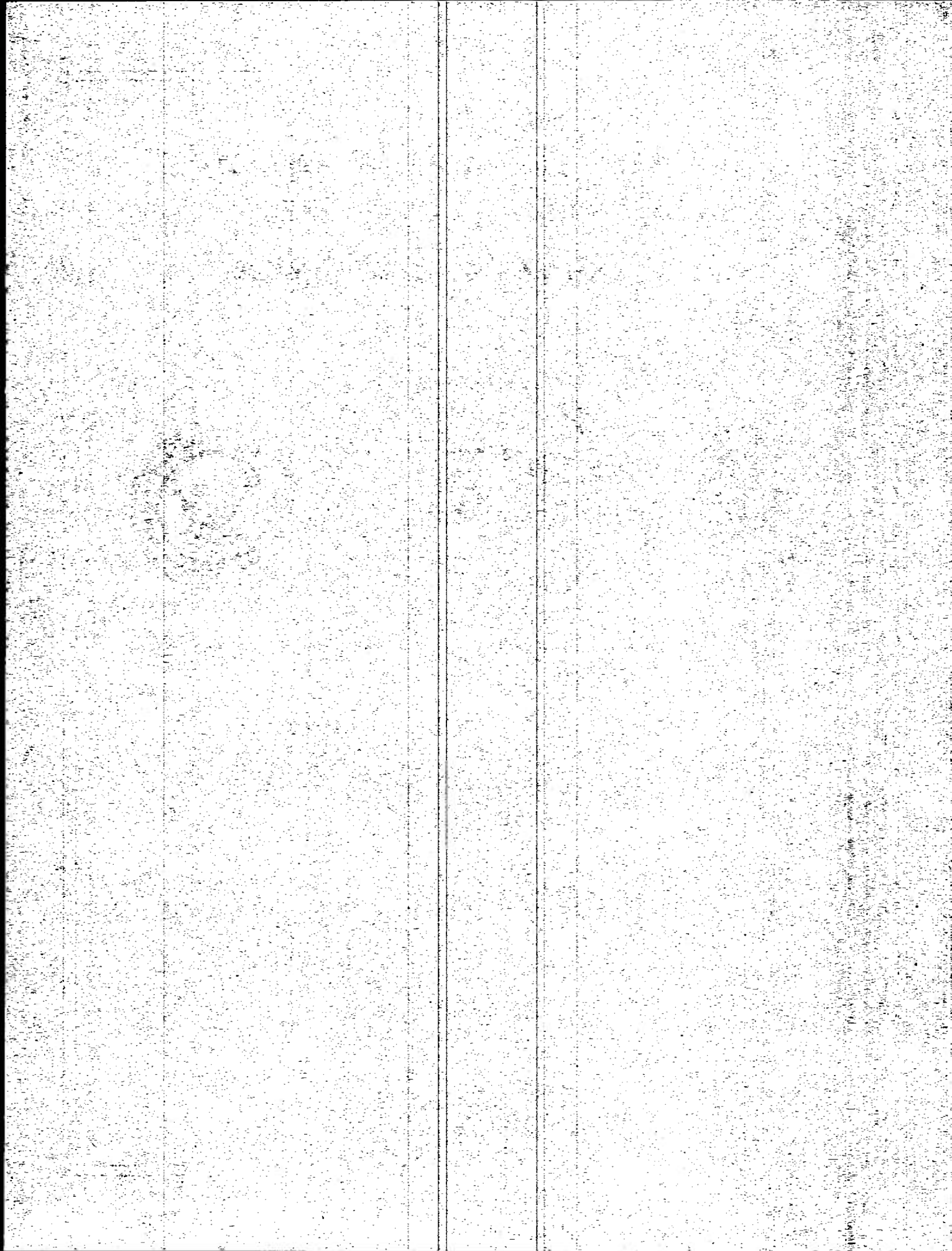
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Bone CO₂-titration curves in acute hypercapnia obtained with a modified titration technique

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PASQUALE, STEPHEN M., ARTHUR A. MESSIER, MICHAEL L. SHEA, AND KARL E. SCHAEFER. *Bone CO₂-titration curves in acute hypercapnia obtained with a modified titration technique*. J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. 48(1): 197-201, 1980.—Bone CO₂-titration curves were obtained in mature rats weighing 500-600 g. Animals were exposed for one hour to 1, 3, 5, 10, or 15% CO₂ in air. Measurements of bone CO₂ were made using a modified titrimetric analysis on fresh and oven-dried samples of paired rat femurs. A manometric method was used for comparison. Arterial blood samples were obtained for measurements of partial pressure of CO₂ in arterial blood (Paco₂). Within the range of environmental CO₂ concentrations studied, a linear relationship was observed between the Paco₂ and the increment in fresh bone CO₂ content. This relationship is defined by the equation: $\Delta \text{fresh bone CO}_2 \text{ (mmol/kg)} = 61.8 + 0.68 \text{ Paco}_2$. The CO₂ increment was confined to a heat-labile, presumably soluble pool comprising 10.5% of the total bone CO₂ content. No change in the water content of the bone was observed as a result of acute CO₂ exposure. The results of this study demonstrate the rapid in vivo CO₂ uptake of bone in response to exposure to increased CO₂ levels.

partial pressure of CO₂ in arterial blood; in vivo uptake of CO₂ by bone; heat-labile bone CO₂ pool

BONE IS THE MAJOR STORE for animal carbon dioxide, comprising about 80% of the total CO₂ storage capacity of the body (11). Bone is also known to be a major reservoir for electrolytes such as potassium, sodium, calcium, and phosphorus. It follows that the bone should play an important role in the maintenance of mineral and acid-base homeostasis. However, little information about CO₂ exchange in hypercapnia and other acid-base balance changes is available in the literature. The few studies that were carried out under conditions of hypercapnia, such as those of Freeman and Fenn (4) and Nichols (8), were limited to the measurement of dried bone CO₂ stores, which only represent fixed carbonates. Neumann and Mulryan (7) were able to show that the CO₂ content of synthetic hydroxyapatite crystals was markedly decreased upon heating. Using [¹⁴C]bicarbonate in vivo, these authors were able to conclude that 60% of bone CO₂ was fixed in the crystal lattice and thereby unexchangeable, whereas 40% of the CO₂ resided in the hydration layer of the crystals where it could possibly exchange with the surrounding fluids. This finding has important implications for the buffering ability of bone. Using a titration method, Bursaux and Poyart (1) deter-

mined the CO₂ content of both fresh and dried paired rat femurs. The loss of CO₂ amounted to 20% of the total CO₂ content and was considered to represent half of the bicarbonate originally present. These authors also demonstrated an increase in bone CO₂ in vivo following mechanical ventilation of eight anesthetized rats for 1 h using 4-6% CO₂ in air. Subsequently, Poyart et al. (9) used a manometric technique to determine bone CO₂ content that provided further evidence for a bone bicarbonate pool. They found that fresh rat bone CO₂ values obtained with the manometric method were higher than those they had previously measured with the titration technique in animals of the same weight.

In our study of the effects of hypercapnia on bone buffering, both the titration and manometric techniques were used to establish a reliable method for the measurement of bone CO₂ content. A modified titration technique produced the best results in our hands.

The in vivo relationship between partial pressure of CO₂ in arterial blood (Paco₂) and bone CO₂ in acute hypercapnia is reported herein.

MATERIALS AND METHODS

Separate groups of six mature Sprague-Dawley rats weighing 500-600 g were exposed for 1 h in environmental chambers (Sherer-Gilette) to 1, 3, 5, 10, or 15% CO₂ in 21% O₂ with the balance being N₂. The environmental temperature was kept at 25.6 ± 1°C. The gas mixtures were prepared by mixing proportional amounts of CO₂ with air. CO₂ and O₂ were added from high pressure cylinders. The air within the chamber was recirculated 12 times/min. With this fast and large turnover of chamber air, mixing of CO₂ and air was nearly instantaneous. The CO₂ concentration in the chamber was continuously monitored with a Beckman infrared analyzer and the O₂ concentration was sampled intermittently with a Beckman oxygen analyzer. In the 10 and 15% exposure, the CO₂ concentration was kept within ±0.5%. At lower levels, regulation was to within ±0.2%. O₂ was kept at 21 ± 0.5%. Prior to blood sampling, the animals received pentobarbital 40 mg/kg body wt intraperitoneally and were returned to the exposure chamber. The anesthesia was usually effective in approximately 5 min, at which time the animals were taken out of the chamber and immediately placed under a mask through which they breathed the same gas mixtures to which they had been exposed.

Blood samples were drawn from the abdominal aorta. Blood pH and PCO_2 were determined with an Instrumentation Laboratory blood gas and pH analyzing system. The femurs of both legs were removed, rapidly cleaned, and stripped free of adhering tissues and bone marrow. Specimens of compact bone between 200 and 300 mg were kept on ice for determination of total CO_2 content. The time between procurement and analysis of the fresh samples did not exceed 2 h. Paired specimens were oven-dried to a constant weight at 150°C for 18 h before analysis.

Bone CO_2 , defined as the CO_2 liberated by bone upon complete dissolution in acid medium, was analyzed by indirect titrimetry employing the following modifications of the method of Bursaux and Poyart (1): 1) the acid medium was heated to 65°C to facilitate complete CO_2 liberation in 6 h rather than 18 h; 2) a second U tube containing 4 N HCl was added in series to obviate potential carryover of HCl vapors; 3) $\text{Ba}(\text{OH})_2$ was used as the CO_2 -trapping medium rather than NaOH. This choice was made based on the observation of Davies (3) that BaCO_3 is sparingly soluble and does not precipitate in the pH range of 8–11; 4) the concentration of CO_2 -trapping agent and titrant were decreased by a factor of 50 to achieve greater sensitivity and smaller titration error; and 5) 2-ml aliquots of $\text{Ba}(\text{OH})_2$ were back-titrated with standardized HCl in duplicate to within ± 0.005 ml in narrow-mouthed vials over a time period of no more than 30 s. Preliminary experiments were unable to detect any effect of atmospheric CO_2 on the normality of 0.1 N $\text{Ba}(\text{OH})_2$ in less than 1.5 min. Figure 1 shows the schematic of the equipment used for bone CO_2 analysis. Seven of these apparatuses were arranged in parallel using the same pump system. Two sets of the apparatuses were employed for the measurement of bone CO_2 in control animals, the others served for analysis of bone CO_2 in experimental animals. Accuracy and precision of the analysis were evaluated by 20 replicate determinations

using 20 mg samples of dried CaCO_3 that yield an amount of CO_2 approximately equivalent to a 250-mg bone sample. The individual samples contained 0.200 mmol CO_2 , of which 0.198 mmol CO_2 (99%) was recovered. The relative standard deviation was 2.5%.

Bone CO_2 content was also measured in fresh and dry cortical bone samples (25–35 mg) with the manometric technique described by Poyart et al. (9). Triplicate analysis was performed on every bone. The water content of bone was determined from the weight loss after drying to a constant weight. Samples were weighed to 0.1 mg on an analytical balance. Group means were compared by Student's *t* test using uncorrelated group design.

RESULTS AND DISCUSSION

Manometric technique. By use of Warburg manometers filled with mercury, the size of the bone sample had to be in the range of 25–35 mg. With larger samples the danger existed that the large volume of CO_2 produced would push the Hg out of the manometer. In carefully controlled tests precise amounts of CaCO_3 were substituted for bone samples and CO_2 was generated according to the method of Poyart et al. (9). The results varied greatly up to 10% indicating that the use of Hg in the manometers made it difficult to get an absolutely accurate reading. This problem was compounded by the fact that a small sample was more difficult to handle in a short period of time and would dry out faster increasing the experimental error. The data obtained were generally somewhat higher than those measured with the titration technique in the same samples. We concluded that in our experience the manometric technique was inferior to the modified titration method described above.

Titration method. Results of the experiments in which the titration method was used are presented in Fig. 2. The total CO_2 content of fresh bone (upper graph) and of dried bone (lower graph) are plotted against PaCO_2 meas-

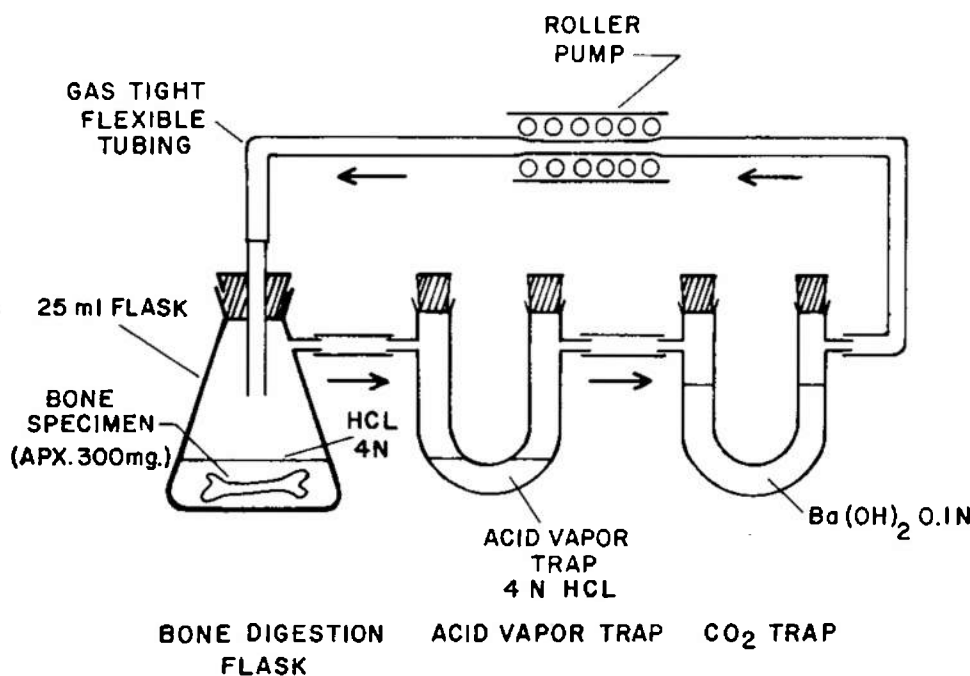


FIG. 1. Equipment used in analysis of bone CO_2 consisting of bone digestion flask, acid vapor trap, and CO_2 trap.

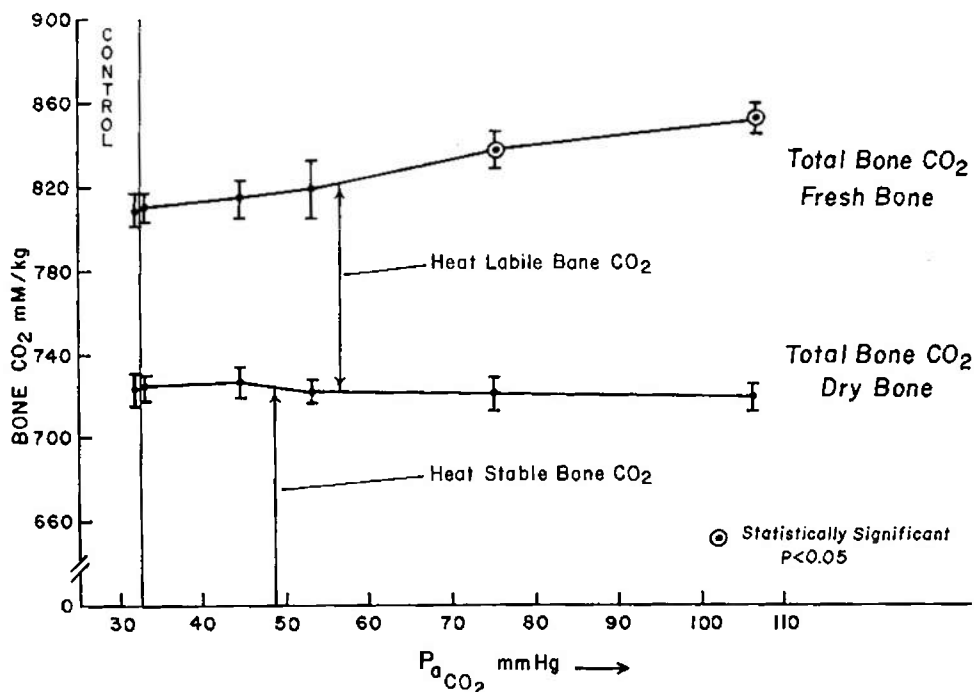


FIG. 2. Relation between bone CO₂ and arterial blood CO₂ tension. Fresh bone CO₂ content (*top curve*); dry bone CO₂ content (*lower curve*). The changes are restricted to fresh bone CO₂ contrast. Difference between fresh bone CO₂ content and dry bone CO₂ content equals heat-labile CO₂ content.

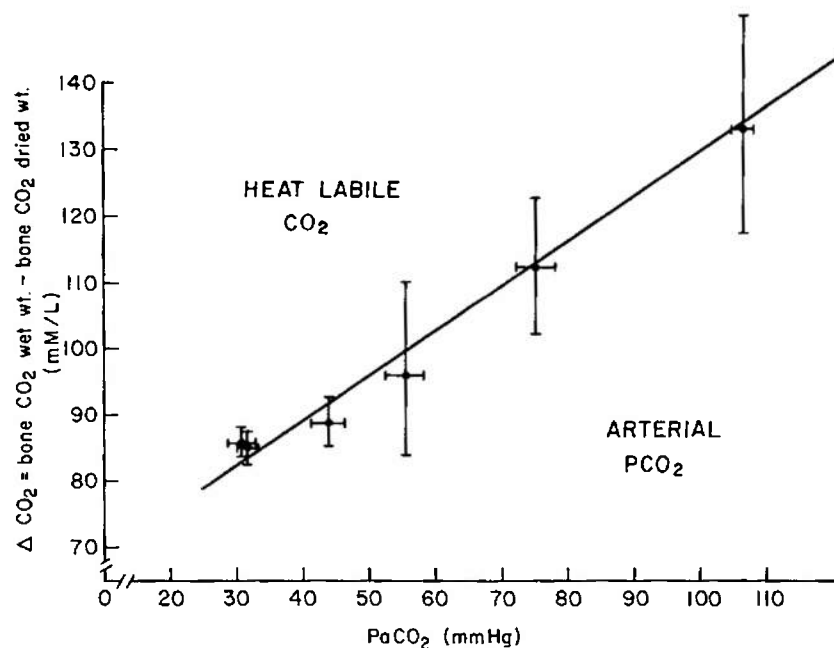


FIG. 3. Increment in heat-labile bone CO₂ as function of arterial CO₂ tension. Each individual point represents mean \pm SE of 6 rats exposed to various levels of hypercapnia for 1 h; total no. of animals used was 36. Calculated regression equation: $\Delta \text{Bone CO}_2 = 61.8 + 0.68 \text{ Pco}_2$ [slope = $+0.68 \pm 0.05$ (SE of estimate); $r = 0.989$; $P < 0.001$; $n = 6$].

ured in the same animals. It should be noted that CO₂ content increased only in the fresh bone samples. In the dried samples, the CO₂ content remained at a constant base-line level, which was 89.5% of the total CO₂ content of fresh control specimens. Based on these data, a heat-labile bone CO₂ pool was defined as the difference between the CO₂ content of fresh and dried bones. It can be seen from Fig. 2 that the increment in bone CO₂ is confined to the heat-labile pool. The absolute change in this pool is therefore equivalent to the absolute change in total bone CO₂ content. Figure 3 shows the regression line for the increment in heat-labile bone CO₂ as a function of PaCO₂.

During the exposure to 15% CO₂, the increase in total bone CO₂ content amounted to 5.4% of the control level.

The heat-labile pool, however, demonstrated a 56.5% increase.

The percent by weight of bone water showed no significant change as a result of CO₂ exposure. These results are summarized in Table 1.

The data on CO₂ content of dry and fresh bone obtained in these experiments before and during exposure to hypercapnic conditions are similar to those reported in the literature. Most of the published values of bone CO₂ content of rats are based only on the analysis of dry bone.

Considerable differences exist in data on CO₂ content of dry rat bone published in the literature. This variation appears to be due to at least three major factors: 1) the age of the animals, 2) the anatomic nature of the bone

TABLE 1. Effects of 1-h exposures to various CO₂ concentrations

%CO ₂ in air	n	Paco ₂ , Torr	Bone CO ₂ , mmol/kg wet wt		Heat-Labile CO ₂		%Bone H ₂ O
			Total (A)	Dried (B)	(A - B)	% Increase	
Control	10	33.1 ±1.9	890 ±9.2	724 ±7.6	85 ±4.9		14.4 ±0.14
1	6	32.1 ±2.4	810 ±5.7	724 ±3.6	86 ±5.3	1.2	13.9 ±0.20
3	6	44.5 ±2.6	815 ±8.7	727 ±5.8	88 ±7.4	3.5	14.8 ±0.15
5	6	53.1 ±2.2	819 ±14.2	723 ±2.4	96 ±14.0	12.9	14.5 ±0.15
10	6	75.2 ±3.0	839* ±3.1	722 ±7.6	117* ±5.3	37.6	14.7 ±0.27
15	6	106.4 ±0.9	853* ±6.6	720 ±15.8	133* ±16.0	56.5	14.0 ±0.19

Values are means ± SE. * Statistically different, $P < 0.05$.

specimen, and 3) the treatment of the bone prior to analysis.

Kramer and Shear (5) showed that the CO₂ content of dry rat bones increases steadily from birth and is not stabilized until approximately 120 days of age. Poyart et al. (9) also observed an age/wt-related increase in CO₂ content of fresh bone. Composition varied in different parts of the same bone. CO₂ and mineral analyses of whole femurs (including both cortical and epiphyses) yielded lower concentrations than those reported for cortical bone alone. Freeman and Fenn (4) reported a value of 582 mmol/kg for animals 86–92 days old (weighing approx 150 g). Nichols (8) employed the method of Danielson and Hastings (2) and found 870 mmol/kg CO₂ for whole ashed femurs of mature rats (290–400 g). Using Warburg nanometers, Poyart et al. (9) measured an average CO₂ content of 751 mmol/kg dry bone in rats weighing between 300 and 352 g. In our studies with the titration technique, the dried bone CO₂ content of mature rats (500–600 g) was found to be 724 mmol/kg. These values agree closely with those of Poyart et al. (9). Larsen et al. (6) using an adaptation of a standard vacuum-line technique measured 500–900 mmol CO₂/kg bone of dogs that is similar to values reported for dry rat bones.

Fresh bone CO₂ content has only recently been studied in conjunction with dry bone CO₂ content. Poyart et al. (9) determined an average of 893 mmol CO₂/kg fresh bone in rats weighing 300–352 g as compared to 809 mmol CO₂/kg fresh bone in rats weighing 500–600 g in our studies. The differences in absolute values of fresh bone CO₂ content obtained with the manometric and titration techniques were much larger than those observed for dry bone. This is most likely related to the small sample size employed with the manometric technique that is approximately 1/10 of that used in the titration technique. It is our experience that handling of such small bone samples (20–35 mg) using the Warburg manometers makes some loss of water practically inevitable which results in a higher measured bone CO₂ content.

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The results of the study of bone CO₂ uptake in acute hypercapnia demonstrated a linear relationship between the arterial PCO₂ and the increment in fresh bone CO₂ content. The slope of this relationship was found to be 0.68 mmol CO₂/kg per Torr. Since the increment of bone CO₂ during acute exposure to different CO₂ concentrations for 1 h was limited to the heat-labile CO₂ pool; further discussion of the nature of this pool is in order.

The chemical nature and location of CO₂ in the bone are at present incompletely defined. Experiments by Neumann and Mulryan (7) have tentatively established at least two CO₂ pools in bone. Based on studies of synthetic hydroxyapatite crystals as well as in vivo labeling experiments with ¹⁴CO₂, these authors concluded that approximately 60% of bone CO₂ was associated with the crystal lattice of bone mineral. Over a 3-wk period, no CO₂ exchange was observed in this pool that was presumed to represent fixed carbonates. The remaining 40% of bone CO₂ was rapidly exchangeable and is presumed to represent HCO₃⁻. They also found that the heating of bone results in a loss of CO₂. Approximately 47% of the ¹⁴CO₂ of the rapidly exchangeable HCO₃⁻ pool was lost upon heating bones from young animals.

Similar findings were obtained by Poyart et al. (1). They produced a steady ¹⁴CO₂ specific activity in the blood after a constant infusion lasting 30 min and found that bone samples heated to constant weight lost more than 50% of the ¹⁴CO₂ activity.

Bursaux and Poyart (1) attributed the CO₂ loss in bone upon heating to the hydration of HCO₃⁻ according to the following reactions: $2\text{HCO}_3^- \rightarrow \text{CO}_3^{2-} + \text{CO}_2 + \text{H}_2\text{O}$. From this reaction as a model, bone HCO₃⁻ could be calculated as twice the CO₂ lost upon heating. The present study established that the CO₂ increment during acute hypercapnia is confined to what is operationally defined as a heat-labile pool. The anatomic nature and chemical composition of this CO₂ space are not definitely known; however, the effect of heat on this pool suggests that it is largely a soluble pool. If it is assumed that this pool is limited to the aqueous phase of bone, and if the model of Bursaux and Poyart cited above for the dehydration of HCO₃⁻ is valid, then using our data for heat labile CO₂ and bone H₂O, the HCO₃⁻ concentration in bone water is calculated to be 1.2 M in control animals with an increase to 1.8 M (33%) after breathing 15% CO₂ in air for 1 h. These concentrations are quite high and suggest that the heat-labile pool may in fact be much larger than the aqueous phase alone.

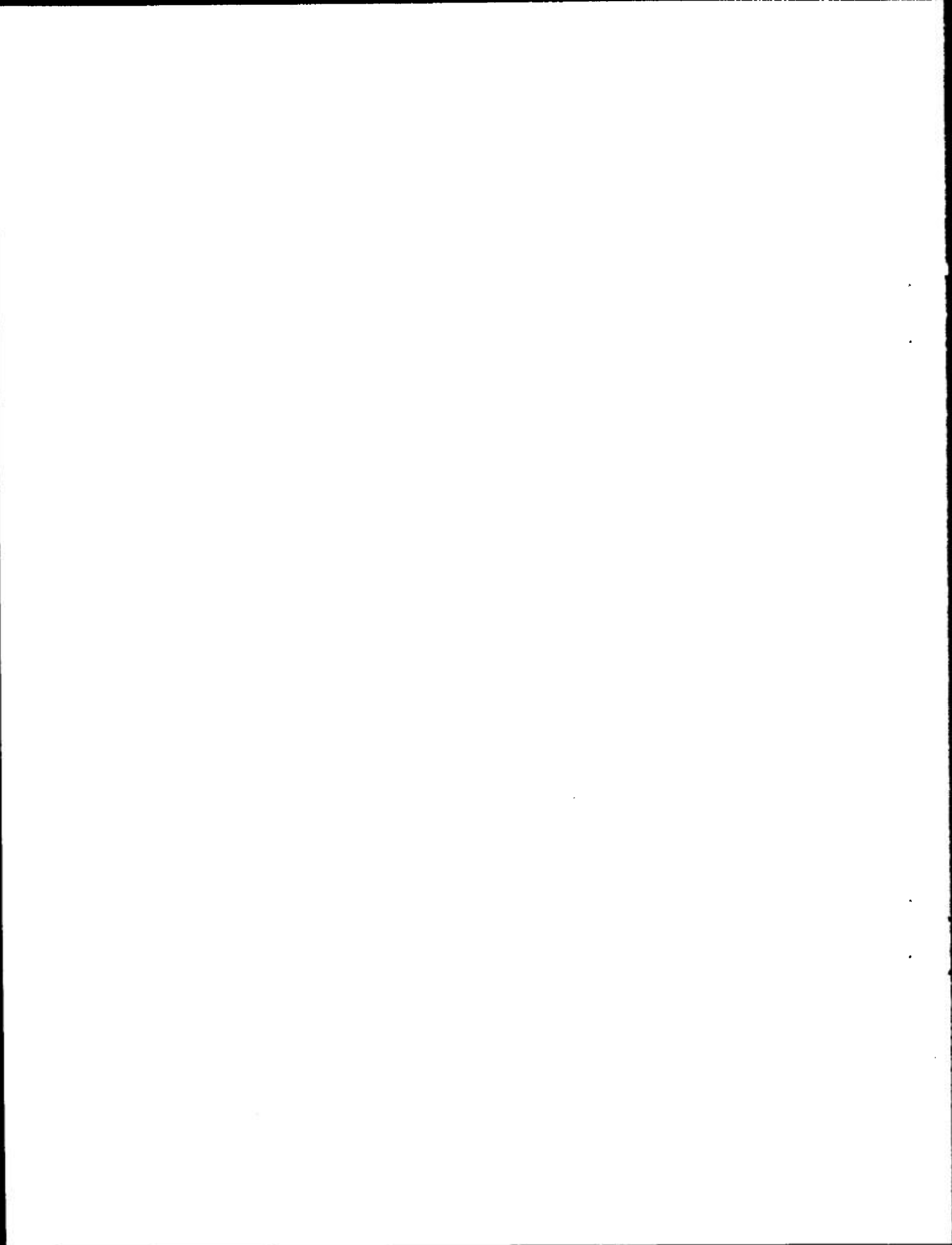
In conclusion, the present study demonstrates the rapid in vivo uptake of CO₂ by bone. The acute increment in CO₂ is shown to be a linear function of Paco₂ and to be limited to a heat-labile pool.

This paper is designated Bureau of Medicine and Surgery, Navy Department Research Work Unit MF51.524.014-9024. The opinions and assertions contained herein are the private ones of the writers and are not to be construed as officially reflecting the view of the Navy Department, the Naval Submarine Medical Research Laboratory, or the Naval Service at large.

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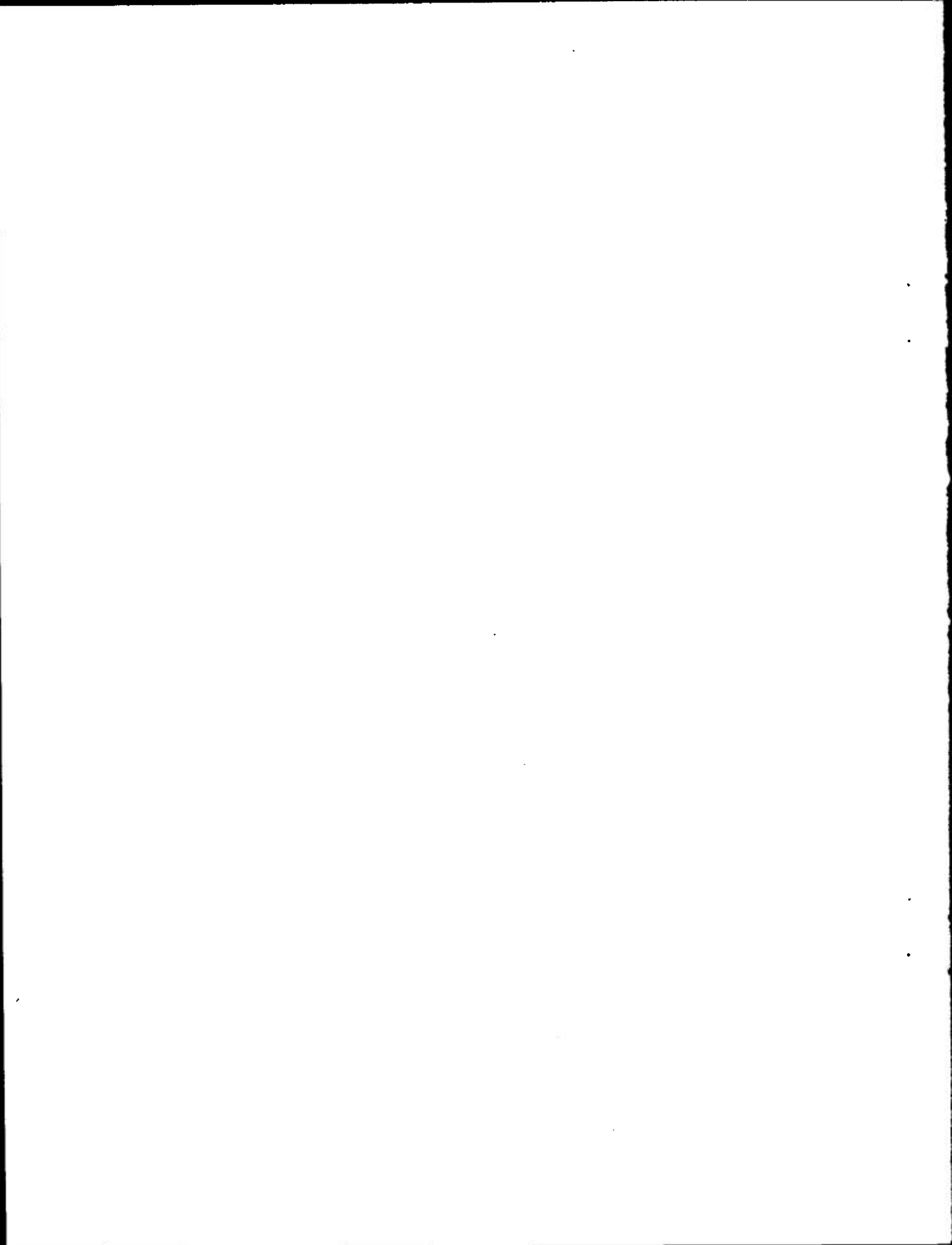


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equation: $\Delta \text{fresh bone CO}_2 \text{ (mM Kg}^{-1}\text{)} = -23.7 + 0.69 P_a \text{CO}_2$. The CO_2 increment was confined to a heat labile, presumably soluble pool comprising 10.5% of the total bone CO_2 content. No change in the water content of the bone was observed as a result of carbon dioxide exposure. The results of this study demonstrate the rapid in vivo CO_2 uptake of bone in response to exposure to increased carbon dioxide levels.